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Identification of an Immunodominant Sequential Epitope in Glycoprotein G of Herpes Simplex Virus Type 2 That Is Useful for Serotype-Specific Diagnosis

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A series of 67 oligopeptides that spanned the open reading frame of herpes simplex virus type 2 (HSV-2) glycoprotein G (gG2) were synthesized and tested for reactivity with 173 serum specimens collected from 117 individuals. The oligopeptides were made as multiple antigenic peptides consisting of four copies of a unique sequence attached to a branched lysine core and separated from the core by four glycine residues. The sera included HSV antibody-negative samples as well as sera from individuals from whom HSV had been isolated. Isolated viruses were typed by indirect fluorescence using a panel of type-specific monoclonal antibodies. One peptide, corresponding to residues 561 to 578 of gG2, did not react with any sera lacking HSV-specific antibodies or with sera from HSV-1-infected individuals, but did react with sera from HSV-2-infected individuals. For sera taken seven or more days after initialclinical lesions, the detection rate of the peptide was 92% (47/51), comparable with the 98% (50/51) of truncated glycoprotein D, a sensitive type-common reagent. We conclude that this peptide, of structure (PEEFEGAGDGEPPEDDDSG$_4$)K$_3$A, is an immunodominant type-specific epitope for human antibodies and should be useful for type-specific serodiagnosis of HSV-2. Surprisingly, the epitope lies within one of the most conserved regions of gG1 and gG2. The test can distinguish an initial HSV-2 infection in the presence of a preexisting HSV-1 infection. ***J. Med. Virol. 56:79–84, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: HSV-2; epitope mapping; immunodominant epitope; glycoprotein G; gG2; serodiagnosis

INTRODUCTION

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are two closely related viruses that infect humans. Most people over the age of 15 have antibodies to HSV, indicating that they have once been infected with and now harbor these viruses. Both viruses produce oro-genital lesion and can also infect the eye, skin, and nervous system. Following primary infection, the virus can enter a latent state in neurological tissue from which it can periodically reactivate to produce recurrent infections. Reactivations are frequently asymptomatic so that viruses can be shed and transmitted in the absence of overt clinical lesions (reviewed by Whitley [1996]).

The prevalence of HSV-2 appears to be increasing and the need for a simple, rapid, and inexpensive serodiagnostic test capable of distinguishing HSV-1 from HSV-2 antibodies was recognized at the recent conference on Epidemiology of Herpes Simplex Virus Infections and Surveillance of Other Sexually Transmitted Diseases in Europe, organized by the World Health Organization [WHO Workshop, 1995]. Most HSV antigens are not suitable for such a test because the type 1 and 2 proteins are generally highly homologous, resulting in extensive cross-reactivity of human antibodies.

Progress toward a type-specific serodiagnostic test has been facilitated by the identification of an HSV-2 glycoprotein, designated gG2 [Marsden et al., 1978, 1984; Roizman et al., 1984] and its HSV-1 counterpart, gG1 [McGeoch et al., 1985; Ackerman et al., 1986; Frame et al., 1986]. Determination and comparison of the DNA sequence of the genes encoding gG1 and gG2 showed the two proteins to have diverged considerably [McGeoch et al., 1985, 1987]. Glycoproteins G1 and G2 contain 238 and 699 amino acids, respectively, and there is no discernible homology in gG1 to residues 20–546 of gG2.

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The two glycoproteins have proved useful for development of various type-specific serological tests, including ELISA, immunodot and Western blotting assays, as well as serum blocking assays using serotype-specific monoclonal antibodies [Lee et al., 1985; Ashley et al., 1988; Johnson et al., 1989; Parkes et al., 1991; Sanchez-Martinez et al., 1991; Ho et al., 1992; Safrin et al., 1992; Boucher et al., 1993; Svennerholm et al., 1994; Kakkanas et al., 1995; Slomka et al., 1995; Hashido et al., 1997]. Of these tests, Western blotting is considered the most reliable; however, the method is cumbersome and not suitable for general screening purposes in other than well-equipped diagnostic laboratories [WHO Workshop, 1995].

To identify immunodominant epitopes on gG2 that might be serotype-specific and useful for a simple and reliable serodiagnostic assay, we synthesized a set of overlapping peptides that spanned the protein, and tested them for reactivity with a panel of well-characterized human sera. Because peptides often lack sensitivity compared with proteins, the gG2 peptides were made as multiple antigenic or branched peptides [Tam, 1988]. Such branched peptides were shown to be capable of detecting lower concentrations of antibodies than could be detected with the equivalent monomeric peptides and, in addition, the sensitivity could be further increased by introducing a four-glycine spacer between the epitope and the polylysine core [Marsden et al., 1992]. Branched peptides have been used previously to develop serological tests that can differentiate between infections with types 1, 2, 3, 4, 5, and 6 of hepatitis C virus (HCV) [Simmonds et al., 1993; Bhattacherjee et al., 1995]. We report here that one such gG2 peptide was found that did not react with any sera lacking HSV-specific antibodies or with sera from HSV-1-infected individuals, but did react with sera from HSV-2-infected individuals. We conclude that this peptide identifies an immunodominant epitope and is useful for type-specific serodiagnosis of HSV-2.

MATERIALS AND METHODS

Sera and Preliminary Serology

A total of 173 serum specimens were collected from 117 individuals. Of these, 155 sera were collected from 100 patients who attended the Department of Genitourinary Medicine at Edinburgh Royal Infirmary in Edinburgh, Scotland. Paired sera were collected from 55 of these patients. A further 18 sera were collected from 18 children, who had been admitted to Yorkhill Sick Children's Hospital in Glasgow, Scotland, with a variety of conditions unrelated to HSV. The HSV-specific antibody titers of sera collected in Edinburgh were determined by complement fixation assay [Ross et al., 1993], while titers in sera collected in Glasgow were screened using an anti-HSV IgG ELISA kit (Enzgnost, Behring Diagnostics, Milton Keynes, Buckinghampshire, UK). The HSV-infected patients from whom sera were collected were adults of the average age of 27 and comprised approximately equal numbers of males and females for each serotype and overall.

Virus Isolation and Typing

All virus isolates were obtained from genital lesions, with a single exception that was isolated from the patient's lip. The viruses were typed by indirect fluorescence using a panel of type-specific monoclonal antibodies (Syva MicroTrack, Behring Diagnostics).

Synthesis of Peptides

A series of peptides, mostly 18-mers overlapping by 10 amino acids, which spanned amino acids 21–699 of the predicted open reading frame of gG2, were synthesized by continuous-flow N^{α}-9-fluorenylmethyloxycarbonyl (FMOC) chemistry (reviewed by Atherton and Sheppard [1989]). Residues 1–20 were not synthesized as these are thought to comprise the signal sequence that is cleaved off the maturing protein [McGeoch et al., 1987]. The peptides were numbered from 1 to 67: the nth peptide comprised amino acids $10n + 11$ to $10n + 28$ in the published amino acid sequence of gG2 [McGeoch et al., 1987], except for peptide 67, which comprised residues 681–699. Peptides were made as multiple antigenic peptides consisting of four copies of each sequence synthesized onto a branched lysine core [Tam, 1988] and separated from it by four glycine residues to increase sensitivity [Marsden et al., 1992]. Such peptides can be represented by the general formula $(peptideGGGG)_4K_3A$. They were made on a Shimadzu multiple peptide synthesizer using procedures described previously [McLean et al., 1991]. Peptides were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) and were judged to be at least 80% pure.

Enzyme-Linked Immunosorbent Assay (ELISA)

Peptides were dissolved in water whenever possible. Peptides not soluble in water were dissolved either in 30% acetic acid (peptides 7, 8, 12, 14, 15, 21, 25, 26, 62) or by bubbling a small volume (<2 ml) of ammonia vapor through the peptide/water suspension, whereupon the peptide formed a clear solution (peptides 16, 17, 18). Solubilized peptides were then diluted in phosphate-buffered saline (PBS) to the concentration indicated in the text and 50 μl were added to wells. Sera were also screened with a truncated version of HSV-2 glycoprotein D that comprised the first 326 amino acids of gD. The truncated glycoprotein (gD2t), purified to homogeneity, was provided by SmithKline Beecham Biologicals (Rixensart, Belgium) at a concentration of 660 μg/ml. It was diluted in PBS and used to coat microtiter wells (Immunolon 1, Dynatech, Billingshurst, West Sussex, UK) with 0.5 μg or 0.25 μg in 50 μl. Both gD2t and peptides were allowed to adsorb to the plate at 4°C overnight. Antigen solution was removed from the plate and unoccupied binding sites on the plate were blocked with phosphate buffered saline (PBS) containing 1% BSA for 1.5–2 hr. Wells were washed six times with 150-mM NaCl containing 0.05% Tween 20 (NaCl-Tween) and were incubated with 50-μl serum, diluted as indicated in the text, for 1.5 hr at room temperature on an orbital shaker. The serum was removed

and wells were washed a further six times with NaCl-Tween. For detection of bound antibody, the wells were incubated in turn with 50 μl of biotinylated sheep antihuman IgG (diluted 1/1000; Amersham, Arlington Heights, IL) and 50 μl of streptavidin-conjugated horseradish peroxidase (diluted 1/1000; Amersham), each for 1.5 hr at 37°C followed by six washes with NaCl-Tween. Chromogenic substrate, o-phenylenediamine dihydrochloride (OPDA) (50 μl) in citrate phosphate buffer (pH 4.0) containing 0.01% hydrogen peroxide was added and, after 10 min, color development was stopped by the addition of 50-μl 2N sulfuric acid. Plates were read on a Titerteck Multiscan plate reader at 492 nm.

RESULTS

Preliminary Screening of All Peptides Against a Subset of the Sera

In an initial experiment, all of the peptides were screened against a set of 24 sera. Four sera were from patients whose isolated virus was found to be type 1 and were antibody-positive, with CF titers ranging from 16 to 256. Fifteen sera were from patients whose isolated virus was found to be type 2 and were antibody-positive, with CF titers ranging from 16 to 256. Three sera were from individuals who had no laboratory evidence of HSV infection, being both virus-isolation- and antibody-negative. Two sera were antibody-negative but from patients from whom virus was isolated: HSV-1 was cultured from one of the patients and HSV-2 was cultured from the other. The sera were also screened against gD2t (500 ng per well) to confirm the presence of HSV-specific antibodies. Wells treated with PBS without any antigen served as controls. In this initial screening, wells were coated with 1.0 μg of peptide and the absorbance values at 492 nm were recorded.

For a peptide to be specific for HSV-2, it should have the following properties. It should react with sera containing antibodies elicited by an HSV-2 infection. In addition, the peptide should not react with sera containing no HSV-specific antibodies or with sera containing antibodies elicited by an HSV-1 infection, in the absence of an HSV-2 infection.

In the absence of any prior data for the reactivity of the peptides, the data were analyzed by two methods. In the first method, a cutoff value of 0.25 absorbance units at 492 nm was chosen on the basis that it could serve as a suitable value for discriminating between HSV antibody-positive and -negative sera. Only peptides 3, 7, 9, 10, 11, 12, 13, 14, 17, 21, 26, 55, 57, 62, 66, and 67 produced absorbance values of >0.25 with all 15 HSV-2 sera, while only peptides 26, 39, 50, 51, 56, 63, and 64 gave absorbance values of <0.25 with all five antibody-negative sera, and only peptides 38, 50, and 55 gave absorbance values of <0.25 with all four HSV-1 sera. Thus, only peptide 55 met all of the criteria required of an HSV-2 antibody-specific reagent. In the second method, the absorbance value of each serum with control (PBS-coated) wells was subtracted from the absorbance values with peptide-coated wells and a cutoff value of 0.1 was chosen. Only peptides 7, 9, 10, 11, 12, 13, 14, 17, 23, 26, 39, 57, 62, 66, and 67 produced absorbance values of >0.1 with all 15 HSV-2 sera, while only peptides 50, 51, 55, 56, and 64 gave absorbance values of <0.1 with all five antibody-negative sera, and only peptides 37, 38, 39, 50, 55, and 64 gave absorbance values of <0.1 with all four HSV-1 sera. Thus, none of the peptides met all criteria. However, of the two peptides (50 and 55) that gave absorbance values of <0.1 with all five antibody-negative and all four HSV-1 sera, peptides 39, 50, and 64 displayed reactivity with 5, 1, and 4, respectively, of the 15 HSV-2 sera, while peptide 55 displayed reactivity with 14 of the 15 sera. Peptide 55, of structure $(PEEFEGAGDGEPPEDDDSG_4)K_3A$, was therefore considered a likely candidate for type-specific serodiagnosis of HSV.

Optimizing the Amount of Peptide 55 and Sera to Be Used

Wells were coated with four different amounts of peptide 55: 5 μg, 1 μg, 100 ng, and 10 ng. Sera were diluted 20-fold followed by six further two-fold dilution. Wells lacking peptide and/or serum served as controls. The results for three HSV-2 sera are shown in Figure 1, panels A, C, and E (CF titers 16, 256, and 16, respectively), and three HSV-1 sera are shown in panels B, D, and F (CF titers <8, 256, and 16, respectively). The HSV-2 sera all showed greater reactivity with gG2/55 peptide-coated wells than with PBS-treated wells, in contrast to the HSV-1 sera, which did not react with peptide 55 above the background levels seen with PBS. With all four peptide concentrations tested, the reactivity of the sera was very similar. Serum A produced an absorbance of greater than 1 when diluted 1:40 or less and appeared to be reaching a plateau. In contrast, both sera C and E showed lower reactivity and the shape of the curves suggested that higher reactivities might be achieved with less dilute sera. To investigate whether even lower amounts of peptide could be used, wells were coated with different amounts, ranging from 100 ng to 5 pg, and tested for reactivity with sera A and E. Below 5 ng per well there was a marked reduction in signal while no response was found below 100 pg (data not shown).

Based on these observations, all sera were then screened on wells coated with 100 ng and 10 ng of peptide 55 and on wells coated with gD2t; the amount of gD2t was reduced to 250 ng per well to reduce the signal to about that seen with the peptide. Sera were tested at dilutions of 5-, 10-, and 20-fold. Wells containing no peptide were included for all dilutions of sera.

Establishing Cutoff Values

The 21 sera that were used as HSV-negative controls comprised three from Edinburgh and 18 from Glasgow. They were judged negative by their lack of reactivity by ELISA (Enzgnost, Behring Diagnostics) or complement fixation assays and by the absence of any clinical symptoms associated with HSV in these patients (see Materials and Methods). The sera were diluted fivefold and

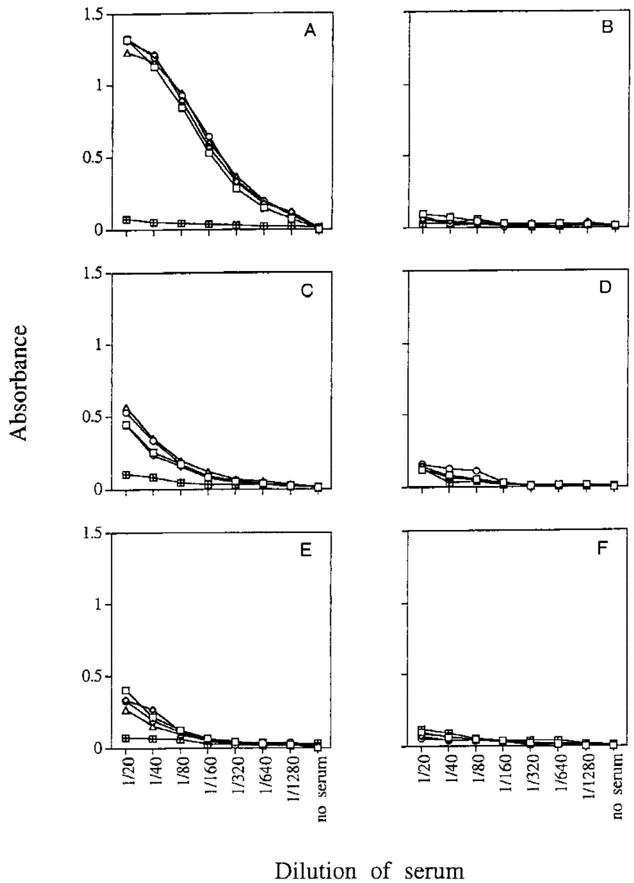

Fig. 1. Optimization of the amount of peptide and sera to be used. Wells were coated with four different amounts of peptide : 5 μg (□), 1 μg (◇), 100 ng, (○), and 10 ng (△). Sera were diluted 20-fold, followed by six further two-fold dilution. Wells lacking peptide (⊟) served as controls. Results for three HSV-2 sera—4395/90 (CF titer = 16), 2226/91 (CF titer = 256), and 5001/90 (CF titer = 16)—and three HSV-1 sera—3401/90 (CF titer = 8), 654/93 (CF titer = 256), and 741/91 (CF titer = 16)—are shown in **panels A, C, E, B, D,** and **F,** respectively.

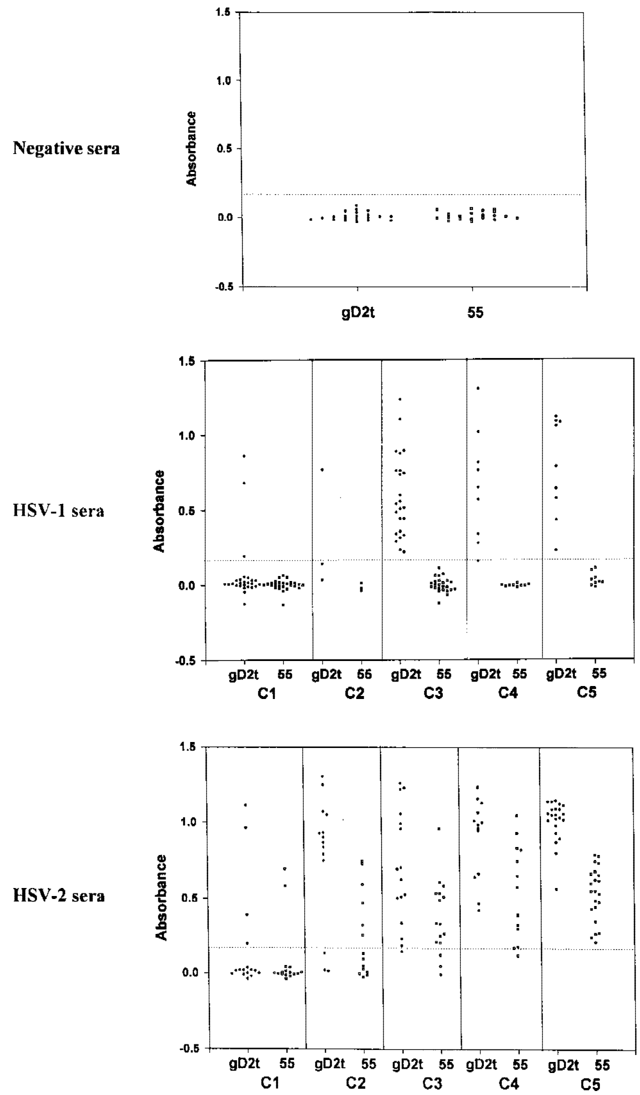

Fig. 2. Screening by ELISA of sera from individuals who were HSV antibody-negative (**upper panel**), infected with HSV-1 (**middle panel**), or with HSV-2 (**lower panel**). Wells were coated with 250 ng of gD2t, 100 ng of peptide 55, or no antigen (PBS control). Sera were diluted fivefold and the ELISA performed as described. The absorbance value in the control wells for each individual sera was subtracted from the values obtained in the wells coated with gD2t or peptide 55 and are shown by closed circles and open squares, respectively. The dotted horizontal line shows the cutoff value derived from the data shown in the figure. C1, C2, C3, C4, and C5 correspond to the five different classes of sera described in the text.

screened by ELISA on wells coated with gD2t (250 ng) or peptide 55 (either 100 ng or 10 ng) or no antigen (PBS control). The data for gD2t and 100 ng of peptide 55 is shown in Figure 2 (upper panel). A cutoff value corresponding to the mean plus 5 times the standard deviation (0.166 for gD2t and 0.167 for peptide 55) was used for subsequent analysis of other sera and is indicated by the dotted line in Figure 2.

Analysis of Sera From HSV-Positive Patients

All sera were screened against wells coated with both 100 ng and 10 ng of peptide 55, 250-ng gD2t, and no antigen (PBS control). Most sera were screened at three different dilutions (5-, 10-, and 20-fold), though some sera were screened using only a fivefold dilution. For each serum, the background absorbance observed without antigen was subtracted from the values obtained for the different antigens. These corrected values constituted the data set.

For analysis, the sera were grouped into five classes. Classes 1 and 2 comprised sera that were collected at the time of presentation from patients with clinical lesions. Class 1 sera had a CF titer of <8, while class 2 sera had CF titers of 8 or greater. Sera comprising classes 3 and 4 were collected between 7 and 20 days (class 3) or greater than 20 days (class 4) after first presentation. Sera in class 5 were from patients who had not presented with a primary lesion and from whom a previous serum had a CF titer of at least 8. Here, a primary lesion is defined as a clinically apparent lesion with a CF titer of less than 8 or a CF titer that rises fourfold within 20 days.

Sera diluted fivefold and screened on wells coated

TABLE I. Reactivity of Sera With gD2t and Peptide 55

	Number of sera scoring positive[b]			
	HSV-1 sera		HSV-2 sera	
Class of serum[a]	gD2t	peptide 55	gD2t	peptide 55
C1	3/26 (12)	0/26 (0)	4/16 (25)	2/16 (13)
C2	1/3 (33)	0/3 (0)	11/14 (79)	6/14 (43)
C3	24/24 (100)	0/24 (0)	15/16 (94)	13/16 (81)
C4	8/9 (89)	0/9 (0)	13/13 (100)	12/13 (92)
C5	9/9 (100)	0/10 (0)	22/22 (100)	22/22 (100)

[a]Classes 1 and 2 comprised sera that were collected from patients with clinical lesions at the time of first presentation. Class 1 sera had CF titer of <8, while class 2 sera had CF titer of 8 or greater. Sera comprising classes 3 and 4 were collected between 7 and 20 days (class 3) or greater than 20 days (class 4) after first presentation. Sera in class 5 were from patients from whom a previous serum had a CF titer of a least 8.
[b]Number of sera giving an absorbance value above the cutoff value of 0.166 (mean + 5 times standard deviation) divided by the number of sera tested. The ratio is expressed as a percentage in parentheses.

with 100-ng peptide gave the least number of false negatives. These results are shown in Figure 2, in which the middle and lower panels represent HSV-1 and HSV-2 sera, respectively, and are summarized in Table 1. The salient features of the data in Figure 2 are as follows. First, there were no false positives, i.e., no HSV-1 serum was reactive with peptide 55. Second, the reactivity of both HSV-1 and HSV-2 sera with gD2t, and HSV-2 sera with peptide 55 was lower for those sera taken at the time of first presentation (classes 1 and 2) than the reactivity of subsequently sampled sera (classes 3, 4, and 5). Overall, 98% of HSV-1 sera (41/42) and 98% of HSV-2 sera (50/51) from classes 3, 4, and 5 reacted with gD2t, while 92% of HSV-2 class 3, 4, and 5 sera (47/51) reacted with peptide 55. Third, all (22/22) HSV-2 class 5 sera reacted with both gD2t and peptide.

CONCLUSIONS

We have identified an immunodominant epitope in gG2 that is completely type-specific for HSV-2 antibodies in human sera. The location within gG2 of the epitope is surprising. As established by McGeoch et al. [1987], gG1 and gG2 have diverged considerably: gG2 with 699 amino acids is almost three times the length of gG1 (238 amino acids). In particular, there are no regions of gG1 that are homologous to residues 20–546 of gG2. We had anticipated that any type-specific epitope would lie within this unique region of gG2. However, the type-specific immunodominant epitope that we have identified lies within one of the most conserved regions of the two proteins. Figure 3 shows the alignment of residues 67–110 of HSV-1 and residues 547–579 of HSV-2. Strikingly, 11 of the 18 amino acids in peptide 55 are conserved in the equivalent region in gG1. We speculate that the type 2 linear sequence may be conformationally constrained by other parts of gG2 to form a type 2-specific structure. Additionally, the heavy N- and O-glycosylation of gG2 [Marsden et al., 1984; Balachandran and Hutt-Fletcher, 1985; Olofsson et al., 1986] may mask epitopes within its unique region. These speculations remain to be investigated.

Peptide 55, which defines the epitope, was of the greatest use in detecting human antibodies after an interval of at least seven days following initial clinical lesions. For this group of patients the detection rate was 92% (47/51) compared with 98% (50/51) for the type-common reagent gD2t. The detection rate was 100% (22/22) for sera from patients from whom a previous serum had tested antibody-positive. It is possible that the detection rate for sera from classes 1, 2, 3, and 4 could be increased by coating the microtiter wells with a greater amount of peptide 55, but this has not been tested.

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HSV-1   PDHTPPMPSIGLEEEEEEEGAGDGEHLEGGDGTRDTLPQSPGPA
        *    * *       ** *******  *  *            *
HSV-2   PGSPAPPPPEHRGGPEEFEGAGDGEPPEDDD           SA
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Fig. 3. Location of the type-specific epitope within gG2. The figure is adapted from Figure 5 of McGeoch et al. [1987] and shows the alignment of residues 67–110 of gG1 and residues 547–579 of gG2, which lie within part of the conserved regions of the proteins. Pairs of identical amino acids are marked with asterisks. The amino acids in the type-specific epitope identified by peptide 55 are underlined.

Our data suggest that with appropriate serum samples, the test can also distinguish an initial HSV-2 infection in the presence of a preexisting HSV-1 infection. Fifty-five individuals who presented with clinical lesions gave at least one serum sample in addition to that provided at first presentation. Such paired sera were obtained from 28 and 27 individuals from whom type 1 and type 2 virus, respectively, were isolated. For four individuals from whom HSV-2 was isolated, the first serum sample, taken at the time of presentation, showed no reactivity with peptide 55 and strong reactivity with gD2t. The subsequent serum samples from these four individuals all showed reactivity with peptide 55 and maintained their strong reactivity with gD2t.

An additional advantage of the method described above for type-specific serodiagnosis is that it needs only 5 μl of serum for each well. Together with the lack of false positives, the possibility of recognizing an initial HSV-2 infection in the presence of a preexisting HSV-1 infection, and the small quantity and low cost of peptide that is needed for each analysis, we believe this

immunodominant peptide will find application as a serodiagnostic reagent for HSV-2 infections.

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NOTE ADDED IN PROOF

Since submitting this manuscript we have become aware of two reports in which overlapping peptides spanning gG2 were used to identify peptides that might be useful for HSV-2 specific serodiagnosis. The first, by Levi et al. (Journal of Clinical and Diagnostic Immunology 3:265–269, 1996) identified three peptides, none of which corresponds to peptide 55. The second, by Liljeqvisr et al. (Journal of General Virology 79:1215–1224, 1998) identified one peptide that partially overlaps the sequence of peptide 55.

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